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SCAI promotes DNA double-strand break repair in distinct chromosomal contexts

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Running title: SCAI is a mediator of several DSB repair pathways

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35 **Summary**

36

37 **DNA double-strand breaks (DSBs) are highly cytotoxic DNA lesions, whose accurate**
38 **repair by non-homologous end-joining (NHEJ) or homologous recombination (HR) is**
39 **crucial for genome integrity and is strongly influenced by the local chromatin**
40 **environment^{1,2}. Here, we identify SCAI (Suppressor of Cancer Cell Invasion) as a**
41 **53BP1-interacting chromatin-associated protein that promotes the functionality of**
42 **several DSB repair pathways in mammalian cells. SCAI undergoes prominent**
43 **enrichment at DSB sites through dual mechanisms involving 53BP1-dependent**
44 **recruitment to DSB-surrounding chromatin and 53BP1-independent accumulation at**
45 **resected DSBs. Cells lacking SCAI display reduced DSB repair capacity,**
46 **hypersensitivity to DSB-inflicting agents and genome instability. We demonstrate that**
47 **SCAI is a mediator of 53BP1-dependent repair of heterochromatin-associated DSBs,**
48 **facilitating ATM kinase signaling at DSBs in repressive chromatin environments.**
49 **Moreover, we establish an important role of SCAI in meiotic recombination, as SCAI**
50 **deficiency in mice leads to germ cell loss and subfertility associated with impaired**
51 **retention of the DMC1 recombinase on meiotic chromosomes. Collectively, our**
52 **findings uncover SCAI as a physiologically important component of both NHEJ- and**
53 **HR-mediated pathways that potentiates DSB repair efficiency in specific chromatin**
54 **contexts.**

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57

58 In response to genotoxic insults such as DNA double-strand breaks (DSBs), eukaryotic
59 cells mount a coordinated DNA damage response (DDR) that activates DNA repair
60 pathways to mitigate the deleterious consequences of DNA lesions^{1,2}. DSBs can be
61 repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR)³.
62 Dysfunctions in DSB repair pathways cause severe hereditary disorders with symptoms
63 including cancer predisposition, neurodegeneration, subfertility, and immunodeficiency⁴.

64
65 The state and organization of chromatin fundamentally influences DSB repair efficiency
66 and pathway choice, and major compositional and structural changes are imposed onto
67 chromatin during DSB formation and repair^{5,6}. DNA damage-induced modifications of
68 chromatin-associated proteins near the lesions enable the accumulation of DNA repair
69 factors at the damage sites^{3,5}. The ATM kinase is a master organizer of this response,
70 phosphorylating substrates including histone H2AX, and this phosphorylation product (γ -
71 H2AX) triggers events that lead to recruitment of the E3 ubiquitin ligases RNF8 and
72 RNF168. Ubiquitin-dependent modification of histones at DSB sites by these ligases then
73 promotes accumulation of DSB repair factors including BRCA1 and 53BP1 to the DSB-
74 surrounding chromatin areas⁷. However, the structure of chromatin can present a
75 substantial barrier to efficient DSB repair. In particular, compacted, transcriptionally inert
76 heterochromatin interferes with the accessibility of repair factors to DNA lesions, and
77 heterochromatin-associated DSBs are generally repaired with slower kinetics than
78 euchromatic breaks⁶. Cells therefore possess multiple factors that remodel chromatin
79 structure to enhance the targeting of DNA repair factors to lesions in heterochromatic
80 regions⁶.

81

82 The chromatin-associated protein 53BP1 is an HR-inhibitory factor that mediates end-
83 joining of unprotected telomeres and other toxic DNA repair reactions⁸. 53BP1 is also
84 crucial for long-range end-joining during V(D)J recombination and immunoglobulin
85 heavy-chain (IgH) class-switch recombination (CSR) in developing lymphocytes;
86 consequently *53BP1*^{-/-} B cells are severely impaired for CSR^{9, 10}. These functions of
87 53BP1 are, to a large extent, mediated by the 53BP1-binding factors RIF1 and PTIP¹¹⁻¹⁵.
88 Finally, 53BP1 has an established, but less well understood, role in promoting ATM-
89 dependent repair of DSBs in heterochromatin. This involves localized phosphorylation of
90 the transcriptional co-repressor KAP1 at S824 in heterochromatin by ATM, which triggers
91 the release of the chromatin remodeler CHD3.1 to enable chromatin relaxation and
92 efficient lesion repair¹⁶⁻¹⁸. Here, we identified the poorly characterized protein SCAI as a
93 mediator of 53BP1-dependent repair of heterochromatin-associated DSBs.
94
95 Using the CHROMASS method for systems-wide profiling of protein recruitment to
96 chromatin templates incubated in *Xenopus* egg extracts that we recently described¹⁹, we
97 observed prominent enrichment of SCAI at DNA damage-containing chromatin along with
98 multiple known DDR components (Figure 1a; S1a,b). SCAI is highly conserved among
99 vertebrates and has been implicated in transcriptional regulation^{20, 21}, but has no annotated
100 domains and shares little sequence homology with other proteins. Using cells expressing
101 GFP-tagged human SCAI at near-physiological levels, we found that SCAI is recruited to
102 microlaser- and ionizing radiation (IR)-generated DSB sites (Figure 1b,c), suggesting it is
103 involved in DSB repair processes. To gain insight into this function, we used quantitative
104 mass spectrometry to identify SCAI-interacting proteins, revealing 53BP1 as well as
105 heterochromatin-associated factors (including the HP1 proteins HP1 β (Cbx1) and HP1 α
106 (Cbx5)) among prominently enriched, prospective SCAI-binding proteins (Figure 1d).

107 Consistently, biochemical fractionation experiments showed that SCAI is predominantly
108 associated with chromatin (Figure S1c). In co-immunoprecipitation assays, SCAI
109 interacted with 53BP1 in an IR- and ATM-stimulated manner, and purified SCAI and
110 53BP1 interacted *in vitro* (Figure 1e,f;S1d), suggesting their interaction is direct and
111 functionally relevant in the context of DSB repair. Knockdown of 53BP1 or its upstream
112 recruitment factor RNF8⁷ strongly attenuated SCAI accumulation at microlaser-generated
113 DSBs, but not *vice versa* (Figure 1b;S1e-i), suggesting that SCAI is recruited to DSB-
114 surrounding chromatin via direct binding to 53BP1, downstream of RNF8/RNF168-
115 mediated histone ubiquitylation. Like ATM inhibition, RNF8 depletion suppressed IR-
116 induced SCAI-53BP1 interaction (Figure 1g), suggesting that the SCAI-53BP1 complex is
117 stabilized once recruited to DSBs.

118

119 Reconstitution of *53BP1*^{-/-} MEFs with different 53BP1 constructs showed that its N-
120 terminus, which undergoes multi-site phosphorylation by ATM to provide binding sites for
121 RIF1 and PTIP¹¹⁻¹⁵, was required for SCAI recruitment to DSB sites (Figure 2a;S2a).
122 Within this 53BP1 region we mapped the SCAI-binding site to amino acids 900-1230,
123 which form part of its ATM phosphorylation domain (Figure 2b). However, unlike RIF1
124 and PTIP, SCAI was recruited to damaged DNA independently of ATM-dependent 53BP1
125 phosphorylation, as expression of a 53BP1 28A mutant refractory to phosphorylation by
126 ATM²² in *53BP1*^{-/-} cells restored SCAI recruitment to DSBs as efficiently as wild-type
127 (WT) 53BP1 (Figure 2a;S2a). Also, downstream effectors of 53BP1, such as RIF1,
128 accumulated at DSB sites independently of SCAI (Figure S2b). Interestingly, while 53BP1
129 depletion markedly impaired SCAI retention at DSB sites, we noted that a subset of cells
130 displayed residual SCAI recruitment to punctate foci along microlaser-generated DNA
131 damage tracks, which colocalized with RPA (Figure 2a,c;S2c). Based on our previous

findings on compartmentalization of nuclear areas flanking DSBs^{3,23}, we surmised that the 53BP1-independent SCAI microfoci might colocalize with RPA-coated single-stranded DNA (ssDNA) regions generated by DSB end resection (Figure S2c). Indeed, these SCAI microfoci in 53BP1 knockdown cells were eliminated upon co-depletion of the key resection factor CtIP, but not by downstream HR factors including BRCA1 and BRCA2 (Figure 2d;S2d). The mechanism underlying SCAI recruitment to resected DSBs may involve its direct binding to ssDNA stretches, as SCAI interacted with ssDNA oligos but not RPA (Figure 1d;S2e). We conclude from these findings that SCAI undergoes enrichment at both the chromatin and ssDNA regions surrounding DSBs, an unusual recruitment pattern observed so far only for BRCA1 and the MRE11-NBS1-RAD50 (MRN) complex²³, key factors in HR.

To understand how SCAI functions in DSB repair, we employed CRISPR/Cas9 technology to generate human cell lines with targeted *SCAI* knockout (KO). While deletion of *SCAI* did not significantly impact cell cycle distribution, *SCAI* KO cells showed reduced cell survival following exposure to IR (Figure 2e,f;S3a,b), consistent with a role for SCAI in promoting DSB repair. Reconstitution of *SCAI* KO cells with full-length ectopic SCAI at near-physiological levels fully rescued this defect (Figure 2e;S3b), demonstrating that it was a specific consequence of *SCAI* ablation. Notably, while 53BP1 loss also sensitized cells to IR as expected, we observed no additive effect of co-depleting SCAI and 53BP1 (Figure 2f;S3c), suggesting that they operate in a common DSB repair pathway. Using quantitative image analysis to monitor DSB repair kinetics through enumeration of γ -H2AX and 53BP1 foci, we observed a significant increase in persistent γ -H2AX and 53BP1 foci in *SCAI* KO cells, which was restored to WT levels by reintroduction of ectopic SCAI (Figure 2g;S3b,d). Using established reporter assays for NHEJ- and HR-

157 mediated repair of DSBs²⁴, we found that SCAI deficiency in human cells led to a
158 pronounced reduction in NHEJ efficiency, while overall HR activity as measured by this
159 system, as well as RAD51 foci formation in response to IR, were not significantly impaired
160 (Figure S3e-i). However, as described below, we obtained evidence that SCAI is important
161 for HR in specific chromosomal contexts.

162

163 To characterize the physiological consequences of SCAI loss, we generated *SCAI* knockout
164 mice and verified complete loss of SCAI protein expression in MEFs from *SCAI*^{-/-} animals
165 (Figure 3a). *SCAI*^{-/-} mice were born at the expected Mendelian frequency (Table S1) and
166 showed no overt developmental or survival defects, demonstrating that *SCAI* is not an
167 essential gene. Moreover, *SCAI*^{-/-} primary MEFs proliferated similarly to WT littermate
168 controls (Figure S4a). To test whether loss of SCAI compromises DSB repair capacity in
169 murine cells, we exposed G2 phase WT and *SCAI*^{-/-} MEFs to low doses of IR and
170 monitored 53BP1 foci clearance over time. Similar to human *SCAI* KO cells, we observed
171 a pronounced persistence of 53BP1 foci at late time points across independent *SCAI*^{-/-}
172 primary MEF lines compared to WT lines (Figure S4b,c). Upon exposure of WT and *SCAI*
173 ^{-/-} mice to whole-body IR, we found that *SCAI*^{-/-} animals died more quickly, ultimately
174 showing an approx. 2-fold survival decrease in both males and females, compared to
175 controls (Figure 3b;S4d,e). Together, these data suggest that SCAI has a physiologically
176 important role in promoting DSB repair efficiency and survival after DNA damage in
177 mammals.

178

179 While 53BP1 is a key DSB repair factor promoting class-switch recombination (CSR) in B
180 cells^{9,10}, we found that SCAI has no obvious role in facilitating this function of 53BP1.
181 Specifically, *SCAI*^{-/-} and control mice displayed comparable splenic B cell numbers and

182 frequencies; moreover, proliferation and class-switching to IgG1 and IgG3 was
183 indistinguishable between *SCAI*^{-/-} and control B cells stimulated *ex vivo* (Figure S4f-i). In
184 addition, *SCAI*^{-/-} mice showed no differences in levels of IgG1, IgG3 or IgM in blood
185 serum compared to control mice (Figure S4j). Instead, full-body necropsies showed that
186 male *SCAI*^{-/-} mice had markedly reduced testis size (Figure 3c,d), suggesting that, unlike
187 53BP1 knockout^{25,26}, ablation of SCAI might result in defective spermatogenesis and
188 subfertility. Indeed, while histological examination of *SCAI*^{-/-} testes showed normal
189 distributions of seminiferous tubules at different stages of spermatogenesis, the lumen of
190 the seminiferous tubules were largely devoid of maturing sperm, with some tubules
191 displaying a Sertoli-cell-only (SCO) phenotype and concomitant expansion of extra-
192 tubular Leydig cells (Figure 3e (I-IV)). As a consequence of these defects, the caudal
193 epididymis of *SCAI*^{-/-} males contained few if any mature spermatids (Figure 3e (V-VI)).
194 Ovaries from *SCAI*^{-/-} and control females were similar in size and shape and both contained
195 fully developed corpora lutea (Figure 3f (I-II)), indicating that overall development of
196 ovary structure and hormonal signaling *per se* were not affected by SCAI loss. However,
197 ovaries from *SCAI*^{-/-} mice contained few or no developing primary follicles (Figure 3f (I-
198 IV)). Consistent with these germ cell maturation defects, we observed substantial
199 reductions in fertility rates of both male and female *SCAI*^{-/-} mice (nearly 3- and 7-fold,
200 respectively) compared to controls (Table S2). Moreover, the few litters generated by
201 *SCAI*^{-/-} female breeding cages were smaller compared to control cages (Figure S4k). We
202 conclude that, unlike loss of 53BP1, SCAI deficiency leads to germ cell development
203 defects and subfertility in both males and females.
204
205 To investigate the underlying cause of defective germ cell development associated with
206 SCAI deficiency, we analyzed spermatocyte spreads from *SCAI*^{-/-} and control testes stained

for meiosis-specific synaptonemal complex markers (SYCP1 and SYCP3). The frequency of meiotic prophase I spermatocytes in leptonema, early zygonema, late zygonema, and diplonema were indistinguishable from controls (Figure 3g). However, *SCAI*^{-/-} testes showed reduced levels of spermatocytes in pachynema and a concomitant increase in aberrant pachynema-like cells characterized by irregular synaptic behavior including gaps, breaks and entangled chromosomes (Figure 3g-i). These data suggest that loss of SCAI leads to impaired meiotic recombination of DNA breaks. Consistently, while the meiosis-specific recombinase DMC1 was loaded normally onto meiotic chromosomes at early stages, DMC1 foci were reduced in late zygonema, early pachynema and on the sex chromosomes of pachynema *SCAI*^{-/-} spermatocytes (Figure 3j;S4I). Nevertheless, *SCAI*^{-/-} spermatocytes form a proper sex body and show normal numbers of diplonema spermatocytes (Figure 3g, i). This suggests that while *SCAI*^{-/-} spermatocytes have a reduced ability to synapse homologs, most are capable of progressing through the mid-pachynema checkpoint. Additionally, late pachynema *SCAI*^{-/-} spermatocytes show a normal frequency of MLH1 foci (Figure 3k), marking sites of future crossovers. Intriguingly, we observed a dramatic reduction in the number of metaphase I cells in *SCAI*^{-/-} testis (Figure 3l). Metaphase I spermatocytes are lost through apoptosis as a consequence of lagging chromosomes, which are primarily caused by the absence of crossing over between homologs²⁷. Our observations suggest that, while crossover designation may be normal in the absence of SCAI, crossing-over itself is disrupted. Thus, loss of SCAI may cause impaired accumulation and/or retention of the HR recombinase DMC1 on meiotic chromosomes and aberrant progression through pachynema, ultimately leading to loss of spermatocytes at metaphase I. These results demonstrate that the germ cell developmental defects and subfertility of *SCAI*^{-/-} mice are at least partially due to aberrant meiotic recombination although relatively mild compared to fully HR-deficient spermatocytes

232 (*SPO11*^{-/-} or *DMC1*^{-/-}) showing severe synapsis and/or pairing defects²⁸. Further supporting
233 a role of SCAI in HR, we observed an increase in chromosomal aberrations in primary
234 *SCAI*^{-/-} B cells compared to controls treated with Olaparib, an established sensitizer of HR-
235 compromised cells (Figure 3m;S4m; Table S3)²⁹⁻³¹. We conclude that SCAI deficiency
236 gives rise to common features of compromised HR-mediated DSB repair in both meiotic
237 and mitotically growing cells.

238

239 While SCAI is dispensable for 53BP1-dependent CSR, we reasoned that it might mediate
240 other 53BP1 functions in DSB repair. The SCAI interactome (Figure 1d) revealed an
241 enrichment of heterochromatin-associated factors including HP1 proteins, which we
242 confirmed biochemically (Figure S5a,b). This raised the possibility that SCAI promotes the
243 function of 53BP1 in repair of heterochromatin-associated DSBs relying on localized,
244 ATM-dependent phosphorylation of KAP1 (pKAP1) in heterochromatin^{6, 16}. Indicative of a
245 heterochromatin-associated NHEJ defect¹⁶, SCAI deficiency in MEFs arrested in G0/G1
246 phase gave rise to an increase in persistent 53BP1 foci after IR (Figure 4a;S5c). Moreover,
247 epistasis experiments using WT and *SCAI*^{-/-} MEFs treated with 53BP1 siRNA or ATM
248 inhibitor showed that loss of SCAI did not exacerbate the DSB repair defect observed upon
249 impaired 53BP1 or ATM function (Figure 4b;S5d), suggesting that SCAI and 53BP1
250 operate in a common pathway to mediate ATM-dependent repair of heterochromatin-
251 associated DSBs. SCAI was recently found to be specifically enriched in pull-downs with
252 histone H3 tail peptides containing trimethylated K9 (H3K9me3)³², the main repressive
253 histone mark in heterochromatin. Indeed, a heterochromatin correlation analysis confirmed
254 that most unrepaired DSBs at late time points in *SCAI*^{-/-} MEFs were associated with
255 H3K9me3-positive chromocenters (Figure 4c;S5e). Similar to *53BP1*^{-/-} MEFs, immediate
256 IR-induced pKAP1, a marker of productive DSB repair in heterochromatin¹⁶, was

markedly reduced after low IR doses in quiescent *SCAI*^{-/-} MEFs (Figure 4d). This effect was partly masked upon increasing IR doses (Figure 4d), as seen also in 53BP1-deficient cells¹⁶. The pKAP1 defect was also evident at persistent heterochromatin-associated breaks, as SCAI knockdown in quiescent 48BR primary human fibroblasts strongly reduced the decoration of γ -H2AX foci with pKAP1 after IR (Figure 4e;S5f). Knockdown of RNF8, an essential mediator of 53BP1 accumulation at DSB sites⁷, phenocopied the effect of SCAI depletion (Figure 4f; S5f). Collectively, these data suggest that SCAI functions downstream of 53BP1 in heterochromatin-associated DSB repair to mediate ATM-dependent KAP1 phosphorylation in repressive chromatin environments.

To further characterize the DSB repair function of SCAI in heterochromatin, we employed a CRISPR/Cas9-based system using gRNAs targeting major satellite repeats to induce heterochromatin-specific DSBs in murine cells³³. The resulting breaks caused rapid accumulation of 53BP1 and GFP-SCAI in DAPI-rich chromocenters corresponding to heterochromatin (Figure S5g). Employing this system to assay signaling from heterochromatin-associated DSBs in WT and *SCAI*^{-/-} MEFs, we found that SCAI deficiency specifically compromised ATM-dependent phosphorylation of KAP1 and H2AX upon DSB formation in heterochromatin, while it had no effect on total levels of KAP1 or 53BP1 accumulation at these structures (Figure 5a-f). Moreover, loss of SCAI did not significantly impact the size and composition of DSB-containing chromocenters, as evidenced by markers such as DAPI, HP1 and H3K9me3 (Figure S5h-k). Consistent with a role for SCAI in promoting overall ATM signaling at heterochromatin-associated DSBs, overexpression of SCAI enhanced ATM-mediated phosphorylations upon formation of such breaks (Figure S5l). Importantly, *SCAI*^{-/-} MEFs did not display obvious ATM signaling defects after IR-induced DSBs, which mostly target euchromatic regions of the

282 genome³⁴ (Figure S5m). Together, these results demonstrate that SCAI functions
283 downstream of 53BP1 to mediate ATM-dependent signaling after DSBs specifically in
284 heterochromatin. 53BP1 promotes repair of heterochromatin-associated DSBs via both
285 NHEJ (in G0/G1 phase cells) and HR (in G2 phase cells)^{16, 35, 36}. Because SCAI deficiency
286 gives rise to a DSB repair defect in both G0 and G2 phase cells, it is possible that SCAI
287 mediates productive DSB repair in compacted heterochromatin via either of these major
288 DSB repair pathways through chromatin remodeling events that facilitate the access of the
289 repair machinery to the lesions.

290

291 Collectively, our data establish SCAI as a physiologically important chromatin-associated
292 component of the cellular machinery that mediates DSB repair in different chromosomal
293 contexts. This involvement minimally includes roles of SCAI in promoting 53BP1-
294 dependent DSB repair in heterochromatin and 53BP1-independent crossover/DSB repair
295 reactions on resected DNA ends during meiotic recombination, likely reflecting its
296 unusual, dual presence at chromatin and end resection-dependent ssDNA regions flanking
297 DSBs, respectively (Figure 5g). Whether SCAI promotes these processes via common or
298 distinct mechanisms, and precisely how it exerts its DSB repair functions at the molecular
299 level, are important future areas of study.

300

301

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327 **Author contributions**

328 R.K.H., A.M., S.L.P., K.T., and K.K. performed the biochemical and cell biological
329 experiments. R.S., A.M. and M.S. carried out mouse experiments. B.R. performed and
330 analyzed the mouse histology experiments. M.R. performed and analyzed the proteomics
331 experiments. R.K.H and M.T. designed and generated CRISPR-based knock-out cell lines.

332 S.O., T.W., R.G. and D.B. generated the SCAI knockout mouse. M.R., M.M., F.C., E.S.,
333 A.G., J.D., N.M., and S.B.-J. designed the experiments and N.M., and S.B.-J. conceived
334 the project and wrote the manuscript. All authors discussed the results and commented on
335 the manuscript.

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338 **Competing financial interests**

339 The authors declare no competing financial interests.

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Figure legends

Figure 1.

455 **SCAI is recruited to DSB-surrounding chromatin via interaction with 53BP1.**

456 **a.** Analysis of protein recruitment to psoralen crosslinked chromatin (PSO) compared to
457 undamaged control. Chromatin templates were replicated in repair-proficient *Xenopus* egg
458 extracts. After chromatin re-isolation, associated proteins were analyzed by mass
459 spectrometry. Maximal protein intensity is plotted against an overall score determined
460 from several independent experiments. The dashed line indicates the significance threshold
461 ($q\text{-value} < 0.01$). Data was replotted from¹⁹. **b.** HeLa cells stably expressing GFP-tagged
462 human SCAI at endogenous levels from a BAC (NFLAP-SCAI) were transfected with
463 control (CTRL), RNF8 or 53BP1 siRNAs. Cells were subsequently subjected to laser
464 micro-irradiation, fixed 1 h later, immunostained with γ -H2AX antibody and counter-
465 stained with DAPI. **c.** U2OS cells stably expressing GFP-SCAI were exposed to ionizing
466 radiation (IR, 5 Gy) and fixed 4 h later. **d.** GFP-SCAI was affinity-purified on GFP-Trap
467 beads from HeLa/NFLAP-SCAI cells, and co-purifying proteins were analyzed by QUBIC
468 mass spectrometry. Intensities and p-values for interacting proteins are shown in a volcano
469 plot. **e.** Chromatin-enriched fractions of U2OS cells exposed to IR and/or ATM inhibitor
470 (ATMi) were subjected to SCAI immunoprecipitation (IP) followed by immunoblotting
471 with antibodies against 53BP1, SCAI and phospho-KAP1. **f.** Interaction between
472 recombinant full-length FLAG-tagged 53BP1 and GST-tagged SCAI was analyzed by
473 GST pulldown followed by immunoblotting with antibodies against FLAG and GST. **g.**
474 U2OS cells stably expressing GFP-SCAI were transfected with control or RNF8 siRNA
475 and exposed to IR where indicated. Chromatin-enriched fractions were subjected to GFP
476 immunoprecipitation followed by immunoblotting with antibodies against 53BP1 and GFP.
477 All scale bars, 10 μ m. Uncropped blots (**e,f,g**) are shown in [Figure S6](#).
478
479

480 **Figure 2.**

481 **SCAI is required for optimal DSB repair.**

482 **a.** 53BP1^{-/-} MEFs were co-transfected with GFP-SCAI and indicated WT or deletion
483 constructs of HA-tagged 53BP1 (Figure S2a), subjected to laser micro-irradiation, fixed 1
484 h later, and immunostained with γ -H2AX or HA antibody. Inserts show larger
485 magnifications of the highlighted regions. **b.** U2OS cells with targeted knockout of 53BP1
486 were co-transfected with GFP-SCAI construct and plasmids encoding indicated Strep-HA-
487 tagged fragments of 53BP1. To analyze SCAI-53BP1 interaction, chromatin-enriched
488 fractions were subjected to Strep pull-down followed by immunoblotting with GFP
489 antibody. N1, 53BP1 residues 250-1972; N2, 600-1972; N3, 900-1972; N4, 1230-1972. **c.**
490 U2OS cells stably expressing GFP-SCAI were transfected with control (CTRL) or siRNA
491 targeting 53BP1 and treated as in (a). 1 h later, cells were pre-extracted and immunostained
492 with RPA antibody. Inserts show larger magnifications of the highlighted regions. **d.** Cells
493 from (c) transfected with indicated siRNAs were processed as in (c) and co-immunostained
494 with RPA70 and γ -H2AX antibodies. All scale bars, 10 μ m. **e.** U2OS WT, 53BP1 KO and
495 SCAI KO cells (Figure S3b, c) were, exposed to increasing doses of ionizing radiation (IR)
496 and plated for clonogenic survival assays. After 14 days, colonies were fixed, stained and
497 counted. Data points indicate the mean from three observations. **f.** Indicated U2OS cell
498 lines (Figure S3b) were transfected with control (CTRL) or 53BP1 siRNAs and were
499 treated and analyzed as in (e). **g.** U2OS cells and derivative cell lines in (e) were fixed at
500 indicated times after exposure to IR (1 Gy) and stained with γ -H2AX antibody. The
501 number of foci per cell was measured by high content microscopy. Centre indicates the
502 median and whiskers the borders of the 95% quantiles. 1000 cells (n=1000 independent
503 measurements) were measured per condition and p-values were calculated from a non-
504 parametric two-tailed Mann-Whitney U test.

505

506 **Figure 3.**

507 ***SCAI* deficiency leads to meiotic recombination defects, germ cell loss and subfertility**
508 **in mice.**

509 **a.** Confirmation of *SCAI* gene disruption by PCR on mouse tails (WT=218 bp, KO=240
510 bp) and immunoblotting of MEFs with the indicated antibodies. Uncropped blots are
511 shown in [Figure S6](#). **b.** 8 Gy whole-body gamma-irradiation of 19 age-matched WT and 22
512 *SCAI*^{-/-} mice. Sex-separated data in ([Figure S4d,e](#)). **c.** Testes from 8-week old WT and
513 *SCAI*^{-/-} mice. Scale bar, 10 mm. **d.** Sizes of n=3 independent testes from (c). **e.**
514 Hematoxylin and eosin-stained sections of testes (I-IV) and caudal epididymis (V-VI) from
515 8-week-old mice. Scale bars I, II, V, and VI, 100 µm; III – IV, 50 µm. **f.** Hematoxylin and
516 eosin-stained sections of ovaries from 14-week-old mice. Bars I and II, 500 µm; III and IV,
517 50 µm. **g.** Spermatocyte spreads from WT and *SCAI*^{-/-} mice stained with SYCP1 and
518 SYCP3 antibodies. 100 cells each from n=3 independent animals were scored and the
519 percentage of cells at each stage plotted. Data were analyzed by Fisher's exact test, two-
520 tailed. **h.** Representative images of spermatocytes stained for MLH1 and SYCP3 showing a
521 pachynema WT cell and a pachynema-like *SCAI*^{-/-} cell. Inset: magnification of the boxed
522 area with entangled chromosomes and loss of synapsis indicated by weaker SYCP3
523 staining. Scale bars, 10 µm **i.** Spermatocytes from (h) were stained for SYCP3 and γ-
524 H2AX to identify chromosome entanglements and sex body. n=3 independent animals
525 were examined and a total of 52 and 58 cells analyzed for WT and *SCAI*^{-/-}, respectively.
526 Statistical analysis was done as in (g). **j.** DMC1 foci were counted at the indicated stages of
527 meiotic prophase I. Pooled cells from three independent animals. P-values were calculated
528 from a Mann-Whitney test. **k.** MLH1 foci counts were plotted as in (j). Pooled cells from
529 three independent animals. **l.** Total number of metaphase cells from one testis. **m.**

530 Metaphase spreads of primary B cells from WT and *SCAI*^{-/-} mice treated with DMSO or
531 PARP inhibitor (PARPi) for 16 h were FISH-stained for telomeric DNA and analyzed for
532 chromosomal aberrations. See [Table S3](#) and [Figure S4m](#). P-value was calculated as in (j)
533 (n=6 independent mice of each genotype). All data points are represented as mean ± SD.

534

535 **Figure 4.**

536 **ATM, 53BP1 and SCAI operate in a common pathway to mediate repair of**
537 **heterochromatin-associated DSBs.**

538 **a.** Independent, immortalized WT and *SCAI*^{-/-} MEF cell lines were arrested in G0/G1 by
539 growing to full confluency. Cultures were mock-treated or exposed to IR (2 Gy), fixed 24
540 h later and stained with 53BP1 antibody. Images were acquired as Z-stacks and the number
541 of 53BP1 foci per cell was counted through the entire nuclear volume. P-value was
542 calculated from a one-tailed t-test using Welch correction (n = 9 independent
543 measurements across 3 MEF lines). Bars indicate mean ± SD. See [Figure S5c](#) for full data
544 set including a 0.5 h time point. IRIF: Ionizing Radiation Induced Foci **b.** Immortalized
545 WT and *SCAI*^{-/-} MEFs were grown to full confluency while transfecting with 53BP1
546 siRNA for 72 h or incubating with ATM inhibitor (ATMi) for 1 h prior to irradiation. Cells
547 were treated and analyzed as in (a), except that they were immunostained for γ-H2AX as a
548 marker of unrepaired DSB (n = 3 biologically independent samples). See [Figure S5d](#) for
549 full data set including a 0.5 h time point. **c.** Immortalized WT and *SCAI*^{-/-} MEFs were
550 treated as in (a), except that cells were co-stained with antibodies to γ-H2AX and the
551 heterochromatin marker H3K9me3 to determine chromatin context (n = 3 biologically
552 independent samples). HC (heterochromatin). See [Figure S5e](#) for analysis of ATM
553 inhibitor treated samples. **d.** Immortalized WT, *SCAI*^{-/-} and *53BP1*^{-/-} MEFs were grown to
554 confluency, exposed to IR (2 or 4 Gy) and harvested at the indicated time points. Lysates
555 were analyzed by immunoblotting with antibodies against total and phosphorylated KAP1.

Uncropped blots are shown in [Figure S6](#). **e.** Quiescent 48BR primary human fibroblasts were transfected with control (CTRL) or SCAI siRNAs, irradiated with IR and fixed after 24 h. Cells were immunostained with antibodies against γ -H2AX and phosphorylated KAP1 (pKAP1), and the relative fluorescence intensities were measured by high content microscopy. Each data point represents one individual IRIF. See [Figure S5f](#) for representative images. **f.** As in (e), except that cells were transfected with control (CTRL) or RNF8 siRNAs. See [Figure S5f](#) for representative images.

Figure 5.

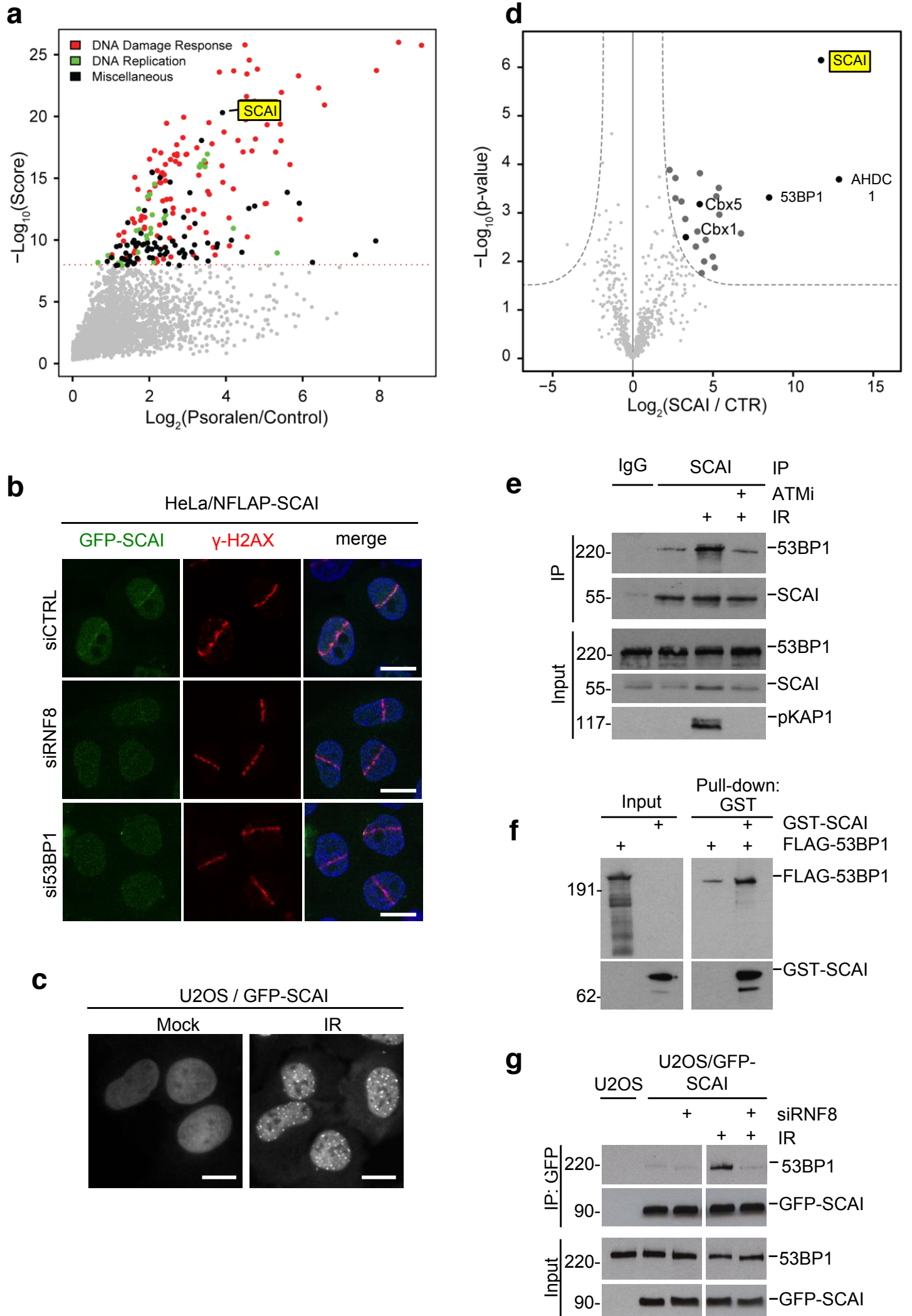
SCAI mediates ATM signaling from DSBs in heterochromatin.

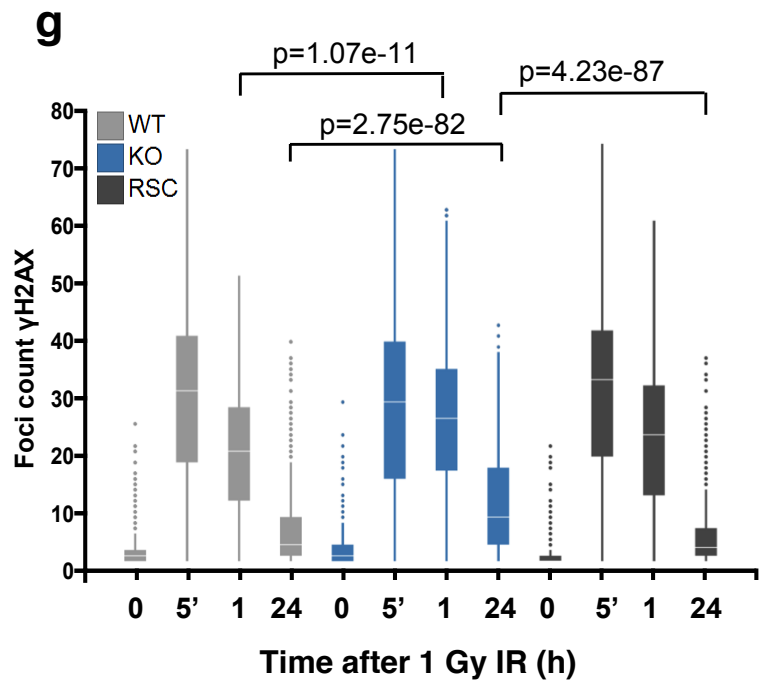
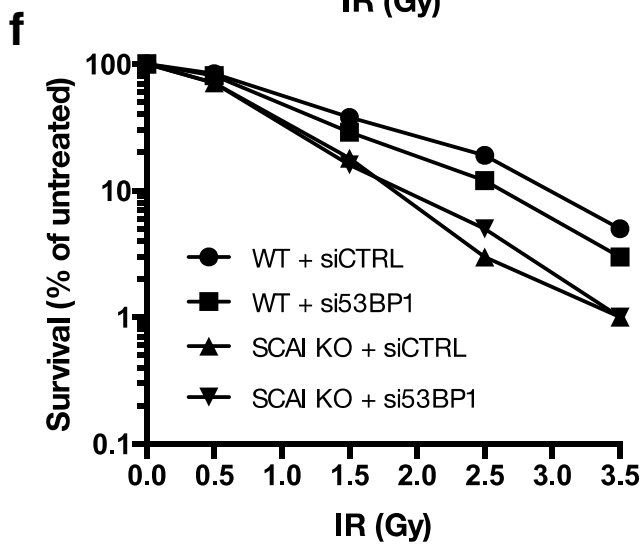
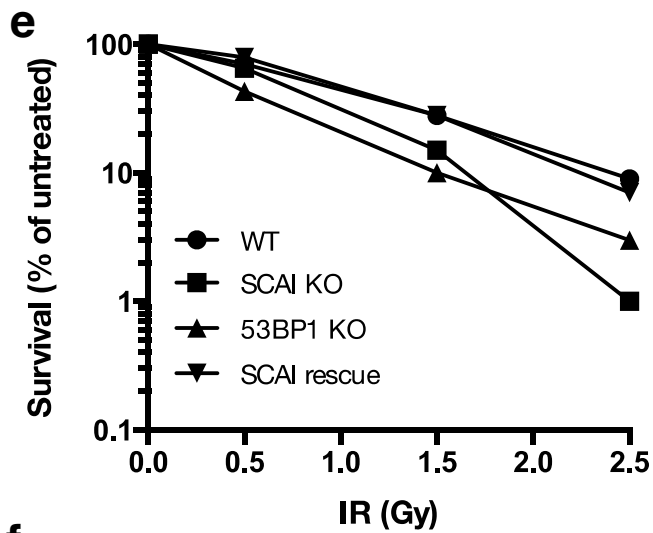
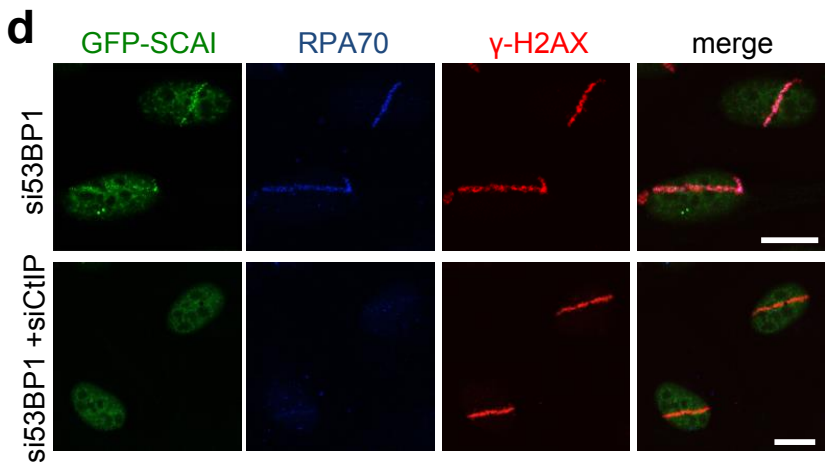
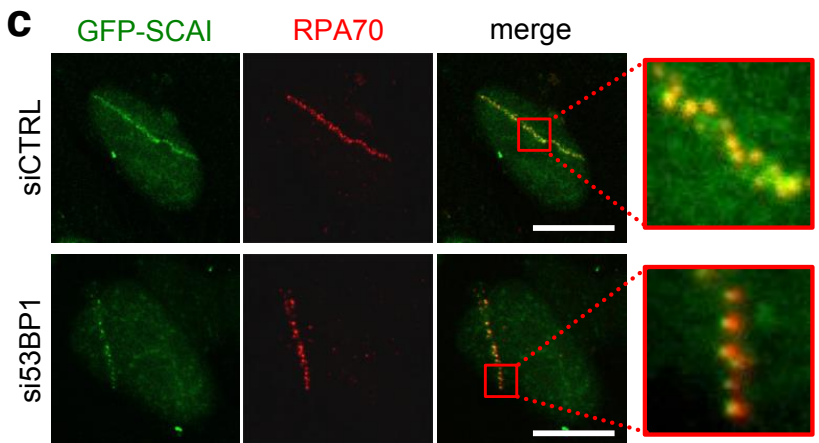
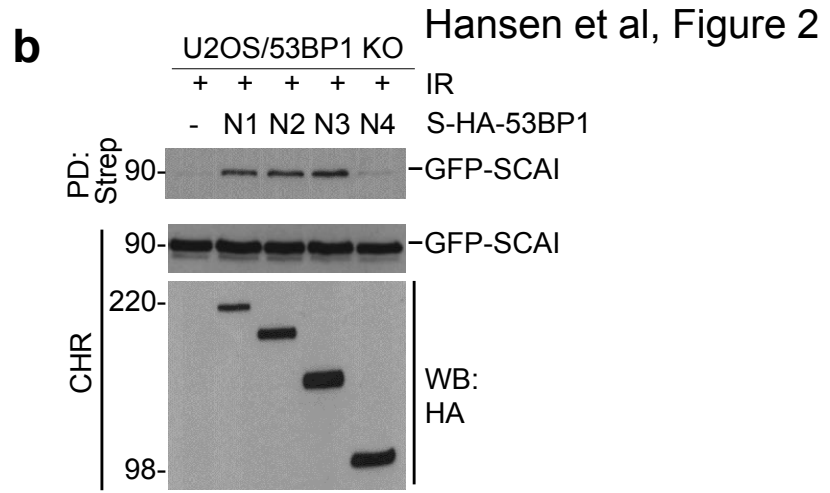
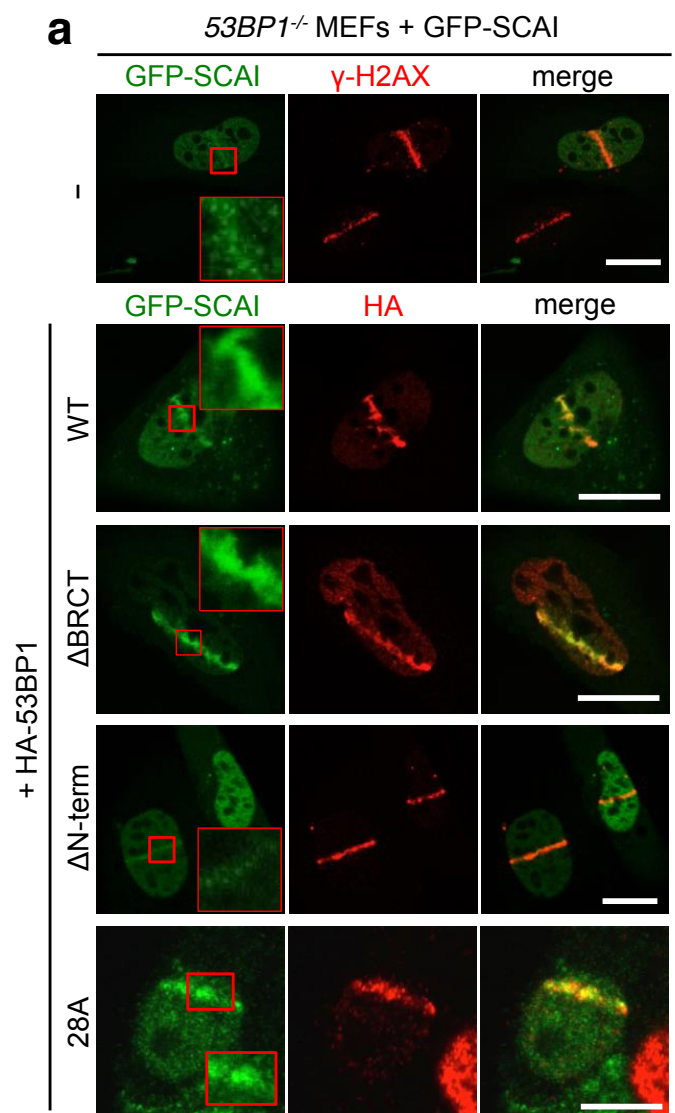
a. Immortalized WT and *SCAI*^{-/-} MEFs were transfected with Cas9-GFP and gRNAs targeting the major satellite repeats to induce CRISPR-mediated DSBs in heterochromatin-containing chromocenters. After 8 h cells were fixed and immunostained with antibodies against phosphorylated KAP1 (pKAP1). Cells were analyzed by high content microscopy using DAPI signal as a mask for chromocenters. P-values were calculated from two-tailed t-tests using Welch correction. Centre indicates the median and whiskers the borders of the 95% quantiles. Y-axis on the left side corresponds to the non-transfected conditions, while y-axis on the right side corresponds to the transfected conditions. (n=200 independent measurements) **b.** As in (a), except cells were immunostained with γ -H2AX antibodies. (n=430 independent measurements) **c.** As in (a), except cells were immunostained with KAP1 antibodies. (n=125 independent measurements) **d.** As in (a), except cells were immunostained with 53BP1 antibodies. (n=550 independent measurements) **e.** Representative images from the experiments in (a) and (b). Scale bars, 10 μ m. **f.** Immortalized WT and *SCAI*^{-/-} MEFs were transfected as in (a) while treated with ATM inhibitor (ATMi) where indicated. Cell extracts were analyzed by immunoblotting with

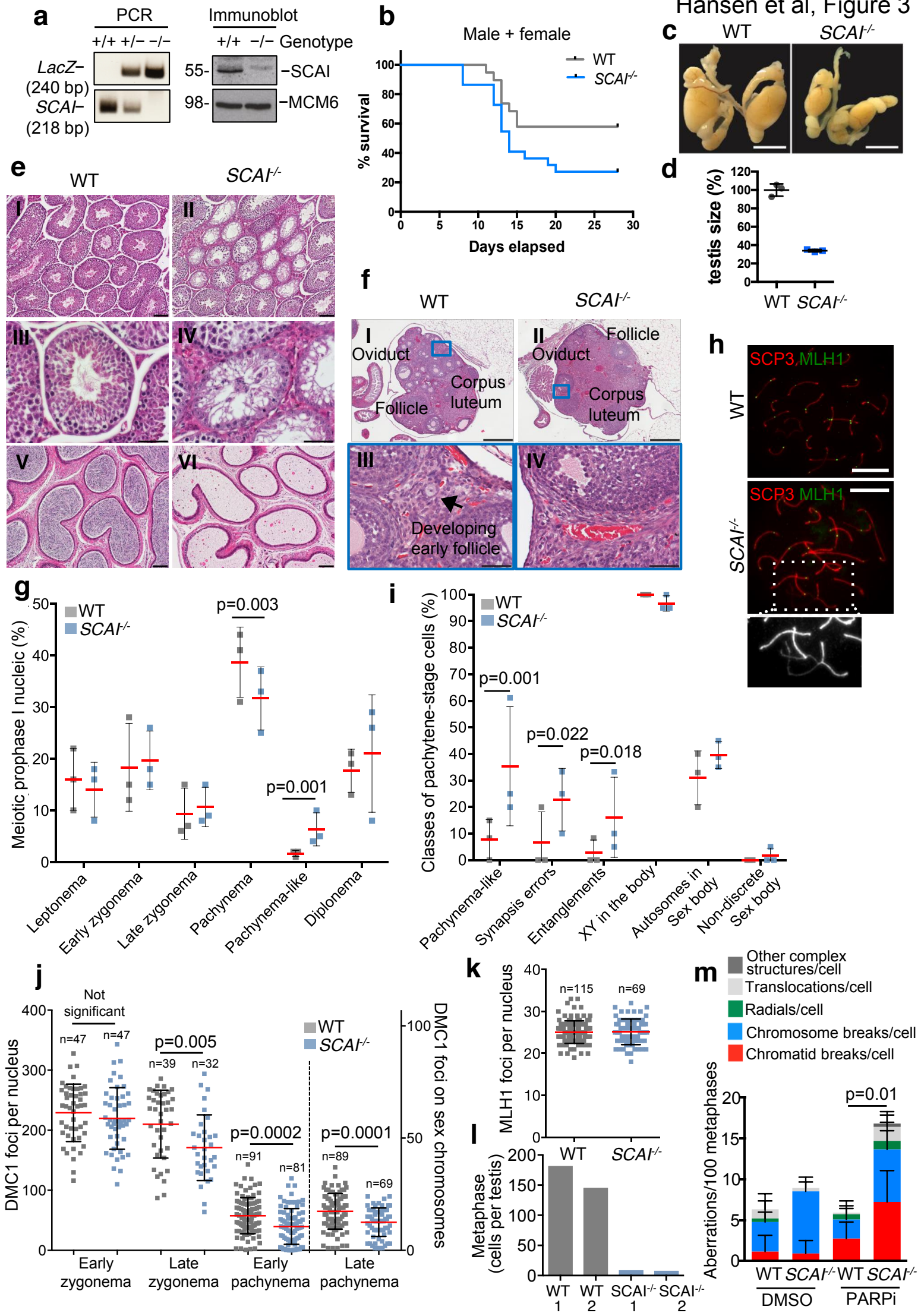
581 indicated antibodies. Uncropped blots are shown in [Figure S6. g](#). Model of SCAI function
582 in DSB repair. SCAI is recruited to DSB-proximal chromatin throughout interphase
583 through direct interaction with 53BP1, promoting 53BP1- and ATM-mediated repair of
584 heterochromatic DSBs. Notably, SCAI is dispensable for other 53BP1-dependent
585 functions, such as immunoglobulin class-switching. During the S and G2 phases of the cell
586 cycle, SCAI also accumulates at CtIP-resected ssDNA regions in a 53BP1-independent
587 manner. From this locale, SCAI supports a subset of HR events, and its deficiency is
588 associated with defects in meiotic recombination and germ cell development.

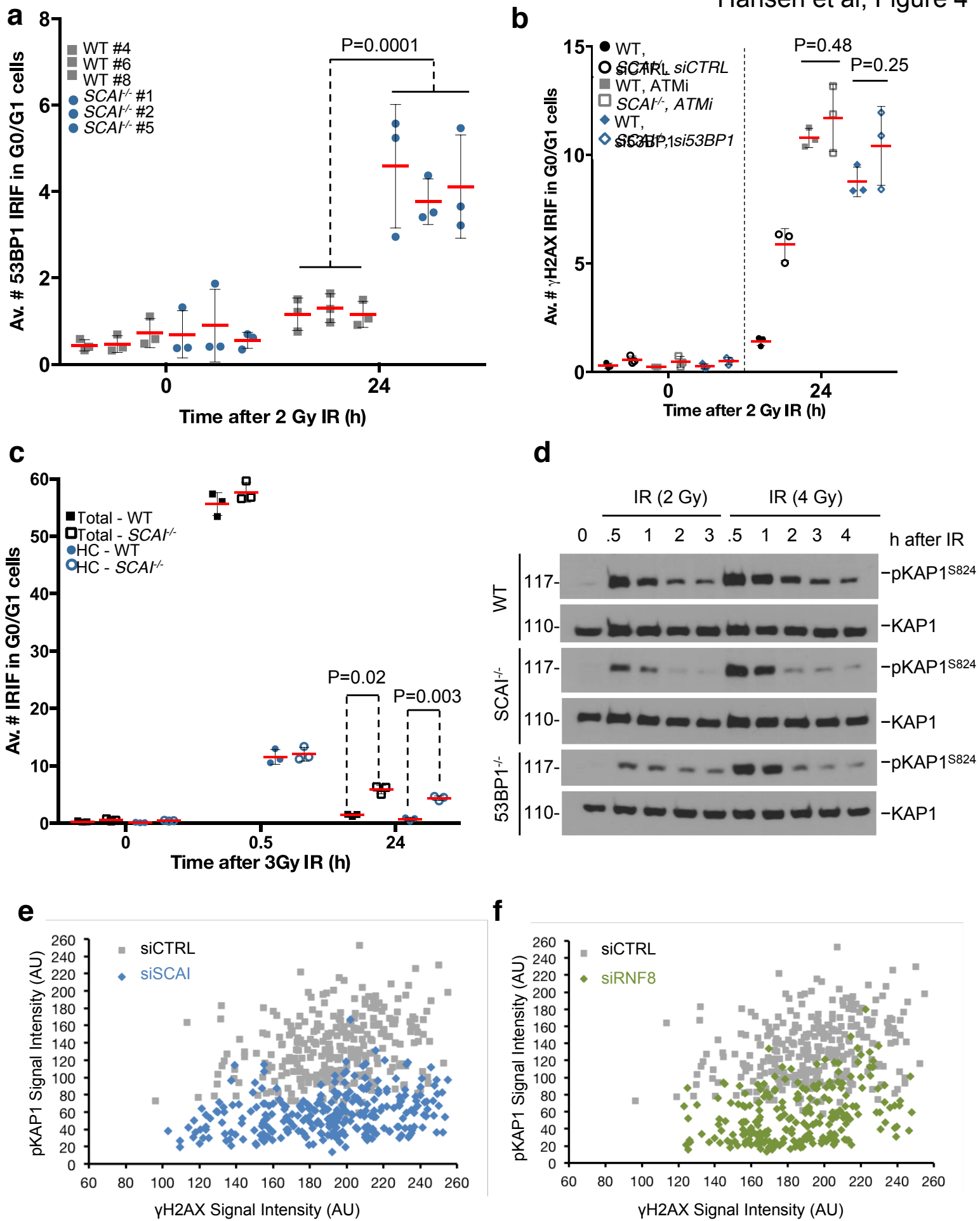
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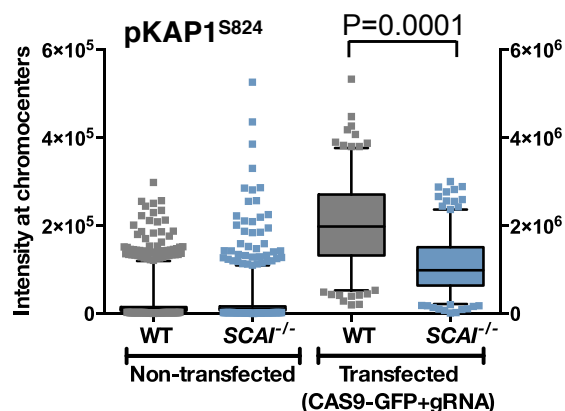
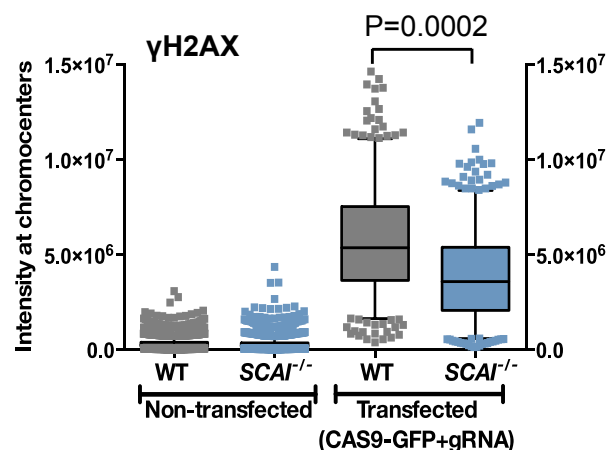
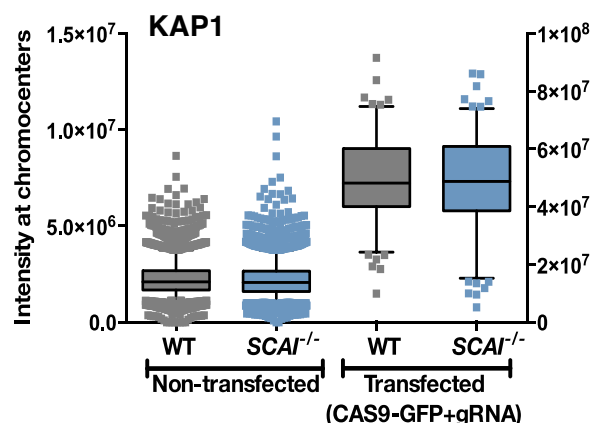
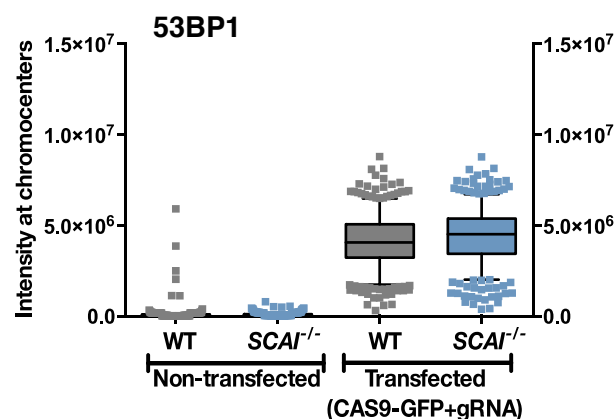
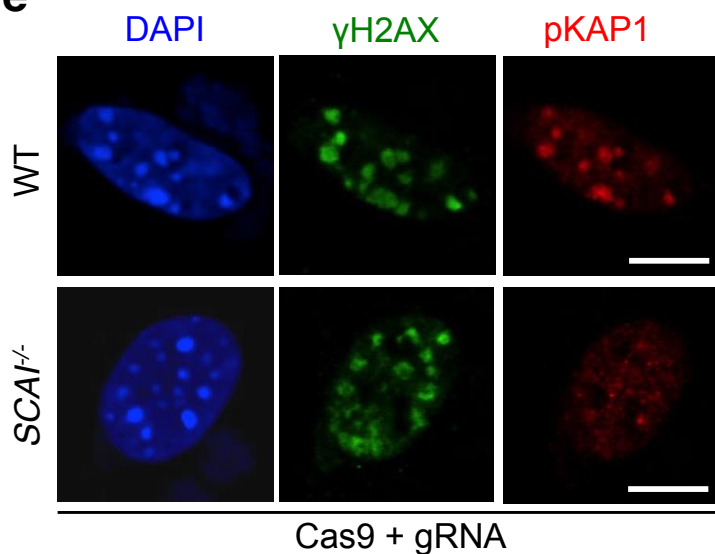
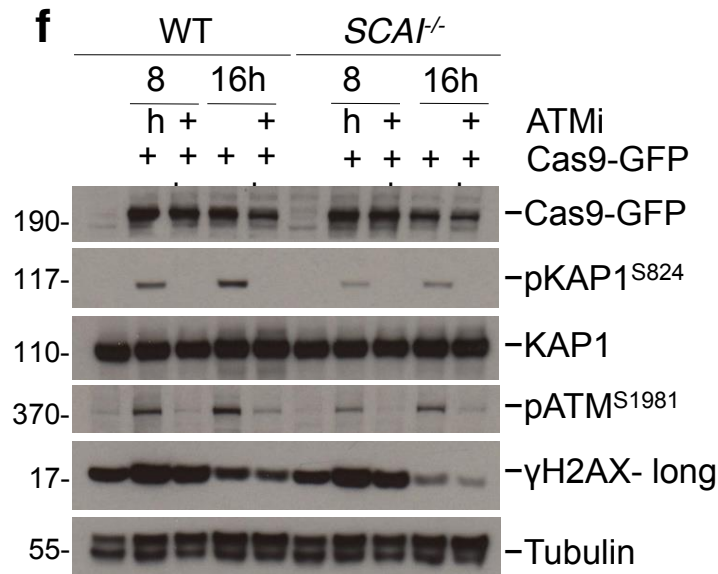
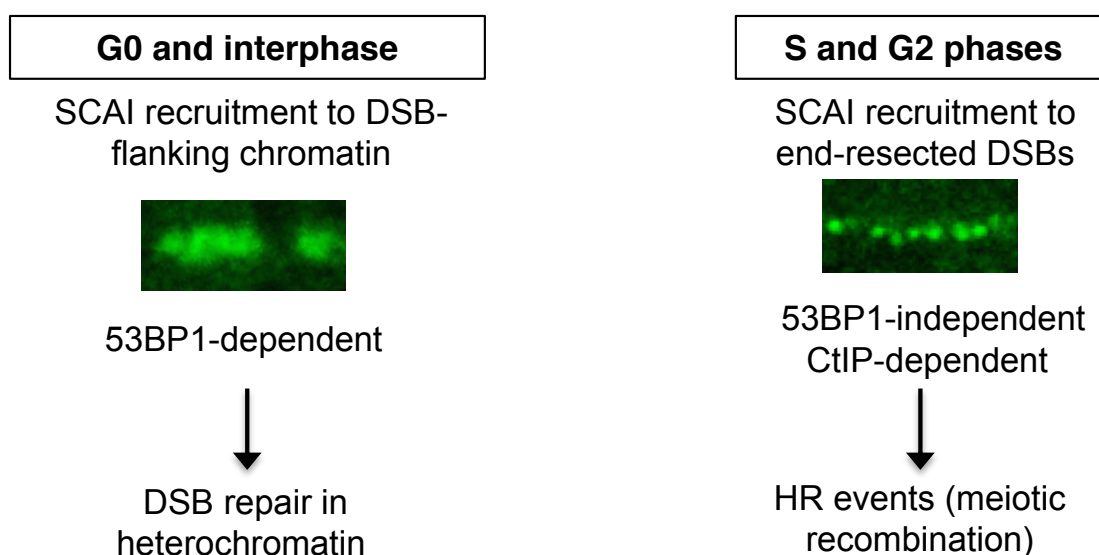
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1 **Methods**

2 *Plasmids and siRNAs*

3 Full-length *SCAI* cDNA was amplified by PCR and inserted into pEGFP-C1
4 (Clontech) and pcDNA4/TO (Life Technologies) containing an N-terminal Strep-HA-
5 tag to generate mammalian expression plasmids for GFP-tagged and Strep-HA-tagged
6 *SCAI*, respectively. The CMK6-HA-53BP1 plasmid was described previously¹⁹.
7 53BP1 N-terminal deletion constructs (N1-N4) were amplified by PCR and inserted
8 into pcDNA4/TO-Strep-HA. Plasmid transfections were performed using GeneJuice
9 (Novagen) or FuGene 6 (Promega) according to the manufacturer's instructions.
10 siRNA transfections were done using RNAiMAX (Life Technologies) according to
11 the manufacturer's instructions. siRNA target sequences used in this study were:
12 Control (5'-GGGAUACCUAGACGUUCUA-3'), *SCAI*(#9) (5'-
13 GAGGCGGAUCCUGUAAUGGUA-3'); *SCAI*(#10) (5'-
14 GGACAGACCUGAAUUGGUA-3'); 53BP1 (5'-
15 GGACUCCAGUGUUGUCAUUUU-3'), RNF8 (5'-
16 UGC GGAGUAUGAAUAUGAA-3'); CtIP (5'-GCUAAAACAGGAACGAAUCTT-
17 3'); BRCA1 (5'-GGAACCUGUCUCCACAAAGTT-3'); RNF8 (5'-
18 UGC GGAGUAUGAAUAUGAATT-3'); and RNF168 (5'-
19 GGCGAAGAGCGAUGGAGGATT-3'). BRCA2 siRNA was an siGENOME
20 SMARTpool from Dharmacon (M-003462-01).
21 Plasmids for generation of *SCAI* knock-out cells by CRISPR/Cas9 were generated as
22 described³⁷. Briefly, *SCAI* gRNA sequences were introduced into pEsgRNA by
23 PCR-based insertion mutagenesis. gRNA sequences used were: *SCAI*#2:
24 GTCTAATAGTGTTGCGTATAAGG (chr9:127757212-127757234); *SCAI*#4:
25 GGCTTGAAGCGCTGGCAAATAGG (chr9:127790713-127790735); 53BP1#1:

26 GCCAGCTCCTGCTCGAAGCTGGG (chr15:43701875-43701897); and 53BP1#2:
27 GTTGACTCTGCCTGATTGTATGG (chr15:43724790-43724812). gRNA targeting
28 major satellite repeats was cloned into vector containing U6 promoter plus followed
29 by a gRNA scaffold. Sequence: Ma-sat#3: GAAATGTCCACTGTAGGACG. Cas9
30 cDNA was amplified from pX330-U6-Chimeric_BB-CBh-hSpCas9 (kind gift from
31 Feng Zhang) and cloned using golden gate cloning into pCX5-CMVp-Cas9-EGFP-
32 SV40p-Puro-pA and pX-86-U6p-gRNA(Ma-sat#3)-CMVp-Cas9-mCherry-SV40p-
33 HygroR-pA plasmids to generate EGFP-tagged and mCherry-tagged Cas9 expression
34 constructs, respectively.

35 *Cell culture and reagents*

36 All standard cell lines were obtained from ATCC and regularly tested for
37 mycoplasma infection. The cell lines were not further authenticated and are not found
38 in the database of commonly misidentified cell lines that is maintained by ICLAC and
39 NCBI Biosample. Human U2OS, HeLa and 48BR cells were cultured in DMEM
40 (GIBCO) containing 10% fetal bovine serum. Mouse NIH-3T3 cells were cultured in
41 DMEM containing 10 % Newborn calf serum. To generate cell lines stably expressing
42 GFP-tagged SCAI, U2OS cells were co-transfected with pEGFP-C1-SCAI and
43 pBabe.puro plasmids and positive single cell clones expanded in the presence of
44 puromycin (1 µg/ml, Sigma). Doxycycline-induced Strep-HA-tagged SCAI cell lines
45 were obtained by co-transfection of pcDNA4/TO-Strep-HA-SCAI and pcDNA6/TR
46 (Life Technologies) and expansion of single cell clones under Zeocin (0.2 µg/ml, Life
47 Technologies) and Blasticidin S (5 µg/ml, InvivoGen) selection. The HeLa/NFLAP-
48 SCAI BAC cell line was a kind gift from Dr. Anthony Hyman (Max Planck Institute
49 of Molecular Cell Biology and Genetics, Dresden, Germany). 53BP1^{-/-} MEFs and
50 reconstituted cell lines were a kind gift from Dr. Andre Nussenzweig (National

51 Institutes of Health, Bethesda, USA). For B cell cultures, resting splenic B cells were
52 isolated from 8-14-week-old WT or *SCAI*^{-/-} mice with anti-CD43 microbeads (anti-
53 Ly48; Miltenyi Biotech #130-049-801) and stimulated to undergo class switching
54 with either LPS (25 µg/ml), α-IgD-dextran (2.5 ng/ml) and RP105 (0.5 µg/ml) for
55 CSR to IgG3 or LPS (25 µg/ml), IL4 (5 ng/ml) and RP105 (0.5 µg/ml) for CSR to
56 IgG1, as described previously³⁸. B cell proliferation was analyzed by CFSE-like
57 labeling using CellTrace Violet proliferation kit (#C34557, Lifetechnologies)
58 according to the manufacturer's instructions. Primary MEFs derived from E13.5 were
59 obtained by intercrossing mice following standard procedures. For immortalization,
60 MEFs were subjected to retroviral infections with SV40LT at passage 2 and cultured
61 in DMEM supplemented with 15% fetal bovine serum (GIBCO), 100 U/ml penicillin
62 and 0.1 mg/ml streptomycin (Sigma). Fibroblast proliferative capacities were assayed
63 by plating passage 2 primary MEF lines (P2). Every 2 days, cells from each dish were
64 trypsinized, counted and replated. Cells were treated with inhibitors targeting ATM
65 kinase (KU60019 (10 µM, Selleckchem) or KU55933 (10 µM, Selleckchem)),
66 proteasome (MG132 (20 µM, AH Diagnostics)) and PARP-1 (Olaparib (1 µM,
67 AZD2281, Selleckchem)). To induce DSBs, cells were exposed to the indicated doses
68 of x-rays using a Y.SMART tube (YXLON A/S, Denmark) at 6 mA and 160 kV
69 through a 3-mm aluminium filter. For high content imaging of RAD51 foci, cells
70 were exposed to IR from a cesium irradiator.

71 ***CRISPR/Cas9 genome editing***

72 *SCAI* or 53BP1 CRISPR knock-out cell lines were generated as described³⁷. Briefly,
73 gRNA plasmids were co-transfected with pBabe.Puro in Cas9-FLAG U2OS SEC-C
74 cells (a kind gift from Dr. John Rouse)³⁷. Cells were grown in DMEM in the
75 presence of doxycycline to induce Cas9-FLAG expression. Subsequently, cells were

grown in the presence of puromycin during clonal selection for 7-10 days.

Knockdown efficiency was validated by qPCR and immunoblotting. Generation of heterochromatin-specific DSBs by Cas9 was achieved by transfecting cells with major satellite-specific gRNA and GFP-mCherry-Cas9 for 8 or 16 h, before pre-extraction in 0.1% Triton/PBS for 30 s followed by fixation in 4% paraformaldehyde/PBS for 10 min.

Mass spectrometry

Analysis of replication-dependent recruitment of proteins to damaged chromatin by means of the CHROMASS method was done as described¹⁹. In brief, psoralen-crosslinked chromatin was incubated in repair-proficient *Xenopus* egg extracts. Chromatin was isolated by sedimentation through a sucrose cushion and analyzed by mass spectrometry.

SCAI-interacting proteins were identified by QUBIC, as described previously³⁹. HeLa BAC cells expressing GFP-tagged SCAI (NFLAP-SCAI) under the control of the endogenous promoter were cultured in DMEM. Pellets from $\sim 10^7$ cells were resuspended in 1 ml lysis buffer (50 mM Tris, pH 7.5; 150 mM NaCl; 5% Glycerol; 1% NP-40; 1 mM MgCl₂) containing 200 U Benzonase (Merck) and EDTA-free complete protease inhibitor cocktail (Roche) and incubated for 30 min on ice. Cell lysates were cleared by centrifugation and GFP-tagged proteins were bound to 50 μ l magnetic beads coupled to monoclonal mouse GFP antibody (Miltenyi Biotec, #130-091-125) for 15 min on ice. Bound proteins were washed three times with 800 μ l ice cold wash buffer I (50 mM Tris, pH 7.5; 150 mM NaCl; 5% Glycerol; 0.05% NP-40) and two times with 500 μ l wash buffer II (50 mM Tris, pH 7.5; 150 mM NaCl; 5% Glycerol). Purified proteins were digested on beads at room temperature by adding 25 μ l digestion buffer (50 mM Tris, pH 7.5; 2 M urea) containing 150 ng Trypsin

101 (Promega) and 1 mM DTT. After 30 min, peptides were eluted by adding twice 50 µl
102 digestion buffer containing 5 mM chloracetamid. After overnight digestion at room
103 temperature, peptides were acidified by addition of 1 µl trifluoroacetic acid and
104 purified on C18 material. Peptides were separated on RP ReproSil-Pur C18-AQ 3µm
105 resin (Dr. Maisch) columns (15 cm) and directly injected into a LTQ-Orbitrap mass
106 spectrometer (Q Exactive, Thermo Scientific, Germany)³⁹. Raw data was analyzed
107 with MaxQuant using the label-free algorithm⁴⁰. ProteinGroups were filtered to have
108 at least three valid values in the LFQ intensities of the SCAI replicates and to be
109 identified by at least two peptides. Missing values in the control pull-downs were
110 imputed by values simulating noise around the detection limit. SCAI interactors were
111 identified by comparing the LFQ intensities in the SCAI and mock pull-downs using a
112 modified two-sided t-test (FDR < 0.01, S0=1, see www.maxquant.org for details).

113 ***Immunoblotting, immunoprecipitation and antibodies***

114 For whole cell extracts, cells were lysed in EBC buffer (50 mM Tris, pH 7.5; 150 mM
115 NaCl; 1 mM EDTA; 0.5% NP-40) or RIPA buffer (1% NP40, 0.5% sodium
116 deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM TRIS pH 8.0) supplemented with
117 protease and phosphatase inhibitors. To obtain chromatin-enriched fractions, cells
118 were lysed in low-salt buffer (10 mM HEPES, pH 7.4; 10 mM KCl; 0.05% NP-40)
119 supplemented with protease and phosphatase inhibitors, and chromatin-associated
120 proteins were released from the pellet by treatment with micrococcal nuclease. Strep
121 pull-downs were done with Strep-Tactin sepharose (IBA) and GFP
122 immunoprecipitation was performed with GFP-Trap agarose (Chromotek). Bound
123 material was resolved on SDS-PAGE and transferred to nitrocellulose membranes.
124 Antibodies used in this study included: rabbit polyclonals to 53BP1 (sc-22760, Santa
125 Cruz, 1:5000 (WB)/1:1000 (IF)), RAD51 (sc-8349, Santa Cruz, 1:150 (IF)), DMC1

126 (sc-22768, Santa Cruz, 1:200 (IF)), RIF1 (A300-569A, Bethyl, 1:200 (IF)), Tubulin
 127 (ab6046, Abcam, 1:10.000 (WB)), Histone H3K9me3 (ab8898, Abcam, 1:500 (IF)),
 128 53BP1 (ab21083 and ab36823, Abcam, 1:1000 (IF); NB100-304, Novus Biologicals,
 129 1:1000 (IF)), SYCP3 (sc-33195, Santa Cruz, 1:200 (IF)), SYCP1 (ab15090, Abcam,
 130 1:200 (IF)), KAP1 (A300-274A, Bethyl, 1:500 (WB)) and phospho-KAP1 (S824)
 131 (A300-767A, Bethyl, 1:1000 (WB & IF)); mouse monoclonals to GFP (sc-9996
 132 (clone B-2), Santa Cruz, 1:500 (WB)), FLAG (F-1804 (clone M2), Sigma, 1:100
 133 (IF)/1:500 (WB)), HA (sc-7392 (clone F-7), Santa Cruz, 1:500 (WB)/1:1000 (IF)), γ -
 134 H2AX (S139) (05-636, (clone JBW301) Millipore, 1:1000 (IF); ab22551 (clone 3F2)
 135 Abcam, 1:1000 (IF & WB)), SYCP3 (sc-74569 (clone D-1) Santa Cruz, 1:200 (IF)),
 136 MLH1 (51-1327GR, (clone G168-15) BD Pharmingen, 1:20 (IF)), BRCA1 (sc-6954
 137 (clone D-9), Santa Cruz, 1:100 (IF)), HP-1 γ (MAB3450 (clone 2MOD-1G6),
 138 Millipore, 1:1000 (WB)), HP-1 β (MAB3448 (clone 1MOD-1A9), Millipore, 1:1000
 139 (WB)), H3K9me2+3 (ab71604 (clone 6F12-H4), Abcam, 1:1000 (IF)), ATM pS1981
 140 (200-301-400 (clone 10H11.E12), Rockland, 1:500 (IF)/1:1000 (WB)) and phospho-
 141 H3 (S13) (ab14955 (clone mAbcam 14955), Abcam, 1:1000 (IF)); rabbit monoclonal
 142 to RPA70 (ab79398 (clone EPR3472), Abcam, 1:1000 (IF)); goat polyclonal to
 143 MCM6 (sc-9843, Santa Cruz, 1:500 (WB)). A sheep polyclonal antibody to SCAI was
 144 generated by immunization with a full-length GST-fusion protein produced in
 145 bacteria (1 μ g/ μ l for IP). Rat monoclonal antibody to SCAI (IH2) was described (1:50
 146 (WB))²¹.

147 ***Immunofluorescence, confocal microscopy and laser microirradiation***

148 Cells were fixed in 4% formaldehyde, permeabilized or pre-extracted prior to fixation
 149 with PBS containing 0.2% Triton X-100 for 5 min or 1 min, respectively, and
 150 incubated with primary antibodies diluted in DMEM for 1 h at room temperature.

151 Following staining with secondary antibodies (Alexa Fluor 488 and 568; Life
152 Technologies) for 30 min, coverslips were mounted in Vectashield mounting medium
153 (Vector Laboratories) containing the nuclear stain DAPI. For detection of nucleotide
154 incorporation during DNA replication, an EdU labeling kit (Life Technologies) was
155 used according to the manufacturer's instructions. Confocal images were acquired on
156 an LSM-780 (Carl Zeiss) mounted on a Zeiss-AxioObserver Z1 equipped with a Plan-
157 Neofluar 40x/1.3 oil immersion objective. Dual and triple color confocal images were
158 acquired with standard settings for excitation of DAPI, Alexa Fluor 488, Alexa Fluor
159 568, and Alexa Fluor 647 dyes (Molecular Probes, Life Technologies), respectively.
160 Image acquisition and analysis was carried out with LSM-ZEN software. Laser
161 microirradiation of cells was performed essentially as described ⁴¹. Imaging of Cas9-
162 induced heterochromatin damage at chromocenters was acquired on Confocal Laser
163 Scanning Microscope TCS SP8 (Leica), using a 63X objective. Spermatocyte spreads
164 were prepared, stained, and scored as previously reported ⁴². Images were acquired on
165 a Zeiss Axio Imager M2 with a Plan-Apochromat 100X/1.4 oil immersion objective.
166 Regarding animals used in spermatocyte spread analyses, age-matched animals were
167 between 18-27 weeks of age, no statistical method was used to predetermine sample
168 size, experiments were not randomized, nor were the investigators blinded to
169 allocation during the experiments or outcome assessment.

170 ***Flow Cytometry and ELISA***

171 Cells were stained with antibodies and measured with an LSR Fortessa cell analyzer
172 (BD Pharmingen) using a DAPI negative live lymphocyte gate. Data were analyzed
173 using FlowJo X 10 software. Antibodies used for flow cytometric analysis included
174 B220 (RA3-6B2), CD19 (1D3), IgM (II/41), IgG1 (A85-1), and IgG3 (R40-82) (BD
175 and eBiosciences). To measure Ig in the blood serum by ELISA, plates were coated

176 with anti-mouse IgM (#406501) or IgG (#1030-01) (Southern Biotechnology
177 Associates, Inc.), and Ig was detected with horseradish peroxidase (HRP)-conjugated
178 goat anti-mouse IgG1 (#1070-05), IgG3 (#1100-05) or IgM (#1020-05) (Southern
179 Biotechnology Associates, Inc.). In all cases, wells were developed with the Ultra
180 TMB peroxidase substrate system (Thermo Scientific) and OD was measured at 450
181 nm using a Fluostar Omega microplate reader (BMG-Labtech). Regarding animals
182 used in FACS and ELISA experiments, animals were between 8-12 weeks of age, no
183 statistical method was used to predetermine sample size, experiments were not
184 randomized, nor were the investigators blinded to allocation during the experiments
185 or outcome assessment.

186 ***Chromosome metaphase spreads***

187 For genome instability analysis, B cells isolated from animals between 8-12 weeks of
188 age were harvested after 3 days in culture stimulated to undergo class switching to
189 IgG1. Metaphase spreads were prepared and processed for FISH analysis as
190 previously described^{11, 43-45}. PARP inhibitor Olaparib (2μM, AZD2281,
191 Selleckchem) was added to cells stimulated *ex vivo* for 16 h and Colcemid
192 (100 ng/ml, Roche) added 1 h before preparation of metaphase spreads, and imaging
193 as described below using a high content microscope. Experiments were performed
194 with the investigator blinded to the group allocation. An assistant labeled the slides
195 and/or dissected spleen/cultured cells before analysis by the investigator, and the data
196 were subsequently related to the identity of the specimens. A total of 165 (WT) and
197 189 (*SCAI*^{-/-}) metaphase spreads from DMSO-treated cells and 452 (WT) and 453
198 (*SCAI*^{-/-}) spreads from PARPi-treated cells were analyzed, across multiple mice, and
199 detailed in the [Table S3](#). Spermatocyte metaphase spreads were prepared as
200 previously described⁴⁶, except a 2.9% isotonic sodium citrate dihydrate solution was

201 used and the slides were stained with Giemsa. Spermatocyte metaphase spread images
202 were acquired on a Zeiss Axio Scope.A1 LED with a Plan-Apochromat 100X/1.4 oil
203 immersion objective.

204 ***Generation of SCAI KO mice and histology***

205 To generate *SCAI*^{-/-} mice, ES cells carrying a targeted allele of the Scai gene were
206 obtained from EUCOMM (allele name: SCAI (tm1a(EUCOMM)Hmgu); clone ID:
207 HEPD0516_1_G04). Correct targeting was verified by PCR using primers spanning
208 the homology arms. PCR fragments from the 5' and 3' end of the targeting construct
209 were cloned into pCR4-TOPO (Invitrogen) and sequenced. Following blastocyst
210 injection, chimeras were mated with C57Bl/6 WT mice and germline transmission of
211 the targeted allele was achieved. The resulting mouse line was crossed with E2A-Cre
212 to remove floxed sequences. Cycling conditions for genotyping PCR were: 94 °C (60
213 s), 60 °C (90 s), 72 °C (120 s), 32 cycles, 72 °C (10 m). Primers
214 LacZ_EUCOMM_for03 (5'-ccagttcaacatcagccgctacagtc-3') and
215 SV40_EUCOMM_rev01 (5'-ctagagcttagatccccctgcc-3') yield a 240-bp product
216 specific for the targeted allele and primers mSCAI_for01 (5'-
217 ccagcacttgggaggcagagac-3') and mSCAI_rev01 (5'-gcagctaaggatagacgatcatagcag-3')
218 yield a 218-bp product for the WT allele.

219 All animal experiments were approved by the Department of Experimental Medicine
220 (University of Copenhagen), the Danish Working Environment Authority, the Danish
221 Animal Experiment Inspectorate, and the MDACC Institutional Animal Care and Use
222 Committee (IACUC).

223 Testes and ovaries from WT and *SCAI*^{-/-} mice were fixed in 10% formalin, and
224 paraffin sections were stained with hematoxylin and eosin. Images were acquired with
225 an inverted microscope (Axiovert 200M; Carl Zeiss, Inc.) equipped with a 10x NA

226 0.45 objective lens (Plan-Apochromat; Nikon) and a color charge-coupled device
227 camera (Axiocam MRc5; Carl Zeiss, Inc.) using AxioVision software (version
228 4.6.3.0; Carl Zeiss, Inc.). Male animals used for histology were 8 weeks old and
229 female animals were 14 weeks old. Similar results were obtained from at least 3 mice
230 of each genotype. For histological analyses, no statistical method was used to
231 predetermine sample size, experiments were not randomized, nor were the
232 investigators blinded to allocation during the experiments or outcome assessment.

233 ***Whole-body irradiation of mice***

234 Age-matched male and female WT and *SCAI*^{-/-} mice were subjected to whole-body
235 gamma-irradiation with a one-time dose of 8 Gy of gamma-rays from a Gammacell
236 40 Exactor Cs137 source and were carefully monitored every day to assess
237 survival. Post-irradiation, the mice were put on antibiotic water for the duration of the
238 study (0.1mg/ml Ciproxin). The experiment with male mice was performed twice
239 with a reproducible result (Figure. S4d) and the experiment with females was
240 performed once (Figure. S4e). Figure. 3b represents the total data of all 3
241 experiments. A scoring sheet used by the animal care-taker was generated to carefully
242 monitor weight-loss, abnormal posture, and lack of movement/lethargy on a daily
243 basis. Animals were euthanized by the care-taker before severe distress/suffering was
244 observed, as determined by the scoring system. As such, the care-takers were blinded
245 to allocation of the genotypes during the experiments and informed the investigator of
246 the data. All remaining animals in the experiment were euthanized by day 28 post-
247 irradiation. WT and *SCAI*^{-/-} mice were age-matched (male exp. 1: 17-27 weeks of age,
248 male exp. 2: 14-42 weeks of age, female exp.: 17-64 weeks of age), no statistical
249 method was used to predetermine sample size, and experiments were not randomized.
250

251 ***High content microscopy and image analysis***

252 Quantitative image-based cytometry (QIBC) for measurement of fluorescence
253 intensities was done as described previously^{47, 48}. The images were obtained with a
254 40x 0.95 NA, FN 26.5 (UPLSAPO40x) dry objective, a quadruple-band filter set for
255 DAPI, FITC, Cy3 and Cy5 fluorescent dyes, a MT20 Illumination system and a
256 digital monochrome Hamamatsu C9100 EM-CCD (electron-multiplying charge-
257 coupled device) camera. Camera resolution is 200 nm x 200 nm per pixel (binning 1,
258 40x). Image analysis was performed with Olympus ScanR automated image and data
259 analysis software using standard algorithms for detection of nuclei and sub-objects
260 within nuclei. Typically, 49 images (corresponding to 1500–3000 sub-objects) were
261 acquired under non-saturating conditions for each data point, allowing robust
262 measurements of experimental parameters such as intensities. Automated unbiased
263 image acquisition was carried out with the ScanR acquisition software. Automated
264 detection and imaging of high-resolution images of metaphase spreads were obtained
265 using Olympus ScanR image analysis and Xcellence software. Images for
266 quantification of 53BP1 and γ H2AX foci were acquired with Olympus ScanR image
267 analysis and Xcellence software. Twenty-five images were acquired and at least 2500
268 cells were analyzed per sample. High-throughput analysis of chromocenters and
269 heterochromatin marker intensities after Cas9 damage induction, were obtained using
270 the IN Cell Analyzer 1000 Cellular Imaging System, followed by analysis using
271 Cellomics Cell-Insight software. Briefly, cells were selected based on DAPI-dense
272 regions and cells expressing Cas9-EGFP-gRNA yielding damage-induced
273 53BP1/ γ H2AX pattern at chromocenters were chosen for intensity analysis.

274 ***DSB repair by IRIF enumeration***

275 Immunofluorescence and DSB repair analysis was carried out as described^{16, 17, 35};

276 briefly, cells were fixed in 3% Paraformaldehyde containing 2% sucrose for 10 min,
277 permeabilized for 3 min in 0.2% Triton X-100 in PBS and immunostained for 1 h
278 with primary antibody (diluted in PBS containing 2% BSA), then 30 min with 1:200
279 dilutions of secondary antibodies (in PBS containing 2% BSA). Cells were
280 counterstained with 0.1 µg/ml DAPI to visualize nuclei and were mounted using
281 Polymount G. Samples were imaged with a Zeiss Axio Observer Z1 platform
282 microscope, with a Plan-Apochromat 20x/0.8, an EC Plan-Neofluar 40x/0.75 or a
283 Plan-Apochromatin 63x/1.4 (oil immersion) objective and an AxioCam MRm Rev.3
284 camera. Acquisition and analysis was done with Zen Pro (Zeiss) software. All error
285 bars on DSB repair graphs indicate the standard deviation. DSB repair analysis within
286 regions of heterochromatin was performed as described ¹⁶.

287 *Clonogenic survival assays*

288 Between 250 and 3000 cells were seeded in 6 cm dishes followed by X-ray irradiation
289 the next day as indicated. After 10-14 days cells were stained with crystal violet
290 solution (0.5% crystal violet, 25% methanol) and colonies containing >100 cells were
291 scored. The experiments were carried out in triplicates and the fraction of surviving
292 cells was normalized to the untreated control.

293 *HR and NHEJ reporter assays*

294 NHEJ or HR reporter constructs (kind gift from Dr. Vera Gorbunova, University of
295 Rochester) were digested *in vitro* with HindIII endonuclease. SCAI CRISPR WT or
296 KO cells were co-transfected with RFP and either circular (negative control) or
297 linearized reporter plasmids. Cells were collected 3 days after transfection and
298 analyzed by FACS as described previously ⁴⁹.

299 *Statistics and Reproducibility*

300 All western blots and microscopy experiments shown in figures were successfully

repeated at least 3 times. For statistical testing of parameters where normal distributions and equal variance could be assumed we calculated p-values by the standard students t-test (Figure S3e,f; Figure S4k). In cases where equal variance could not be assumed, we used t-test with Welch correction (Figure 4a,b,c; Figure 5a,b,c,d; Figure S4b,S5c,d,e,h,i,j,k). For data sets where normal distribution could not be assumed, we employed the non-parametrical Mann-Whitney U test (Figure 2g; Figure 3j,k,m; Figure S3d) or Fisher's exact test (Figure 3g,i).

Data availability

The entire CHROMASS mass spectrometry data set has been deposited to the ProteomeXchange Consortium via the PRIDE⁵⁰ partner repository with the dataset identifier PXD000490 (<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX000490>), and was previously published¹⁹. SCAI interactome recorded by Label Free Quantification (Figure 1d) has been deposited with the dataset identifier PXD004912 (<http://proteomecentral.proteomexchange.org/cgi/GetDataset?ID=PX004912>). All other data supporting the findings of this study are available from the corresponding authors upon request.

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